

The M Protein of Vesicular Stomatitis Virus Associates Specifically with the Basolateral Membranes of Polarized Epithelial Cells Independently of the G Protein

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Abstract. Using monoclonal antibodies and indirect immunofluorescence microscopy, we investigated the distribution of the M protein in situ in vesicular stomatitis virus-(VSV) infected MDCK cells. M protein was observed free in the cytoplasm and associated with the plasma membrane. Using the ts045 mutant of VSV to uncouple the synthesis and transport of the VSV G protein we demonstrated that this distribution was not related to the presence of G protein on the

cell surface. Sections of epon-embedded infected cells labeled with antibody to the M protein and processed for indirect horseradish peroxidase immunocytochemistry revealed that the M protein was associated specifically with the basolateral plasma membrane. The G and M proteins of VSV have therefore evolved features which bring them independently to the basolateral membrane of polarized epithelial cells and allow virus to bud specifically from that membrane.

EPITHELIA are formed from sheets of cells connected by a variety of specialized junctions. They play a crucial role in such diverse processes as water and ion balance, information transduction, absorption of nutrients, and excretion of waste products. The polarized nature of the cells making up these tissues is central to their function. Not only is the cytoplasm organized in a highly polarized manner, but the plasma membrane of these cells is divided into two domains containing different component proteins. Almost nothing is known about the mechanisms cells use to initially sort proteins into one or the other of these domains.

Rodriguez-Boulan and Sabatini devised an experimentally manipulatable system to study this sorting. They showed that when enveloped viruses infected the established Madin-Darby canine kidney (MDCK) epithelial cell line, budding of viral particles occurred from only one of these domains (6). For example, influenza virus buds from the apical plasmalemma and VSV buds from the basolateral plasmalemma. Subsequent work demonstrated that the glycoproteins of these viruses are appropriately sorted into either the apical or basolateral domain, respectively (5). More recent work has placed the sorting event at a step after these proteins have traversed the Golgi cisternae and before they reach the cell surface (16, 34, 37, 45, 47, 48).

Much less is known about the sorting of the other viral constituents. Rindler et al. found that although most of the G protein was present on the basolateral plasma membrane of infected MDCK cells, some G protein could be identified on the apical plasma membrane. However, this apical G protein was not sufficient to induce apical budding of VSV (47). Also, Roth and Compans found that the formation of pseudotypes between influenza and VSV was delayed in MDCK

cells (51). Together these data indicate that the polarized budding of enveloped viruses may involve viral components other than the glycoproteins. There is also good genetic and biochemical evidence that the glycoproteins are not solely responsible for determining the site of viral budding. For example, when cells are infected with temperature-sensitive mutants of VSV under conditions which retain G protein in the rough endoplasmic reticulum (RER)¹, there is no budding of VSV into the RER (2, 4). Also, temperature-sensitive class III mutants (affected in M protein function) produce very few virus-like particles (54). Finally, there is no fixed stoichiometry between the glycoprotein and the other structural proteins of VSV. Even in wild-type VSV-infected cells, the relative amounts of the G and M proteins in virions varies sixfold during the course of the infection whereas the proportion of M and nucleocapsid (N) proteins remains constant (31). These findings suggest that the M protein plays an important role in the budding of VSV in all cells and further, that in epithelial cells, the M protein may play a role in the polarized budding of VSV. A corollary of this inference is that the M protein must itself be sorted to the appropriate plasma membrane domain in infected MDCK cells.

M protein differs from the model proteins studied to date in that it is a peripheral membrane protein synthesized on ribosomes that are not associated with the endoplasmic reticulum or any other cellular membrane system (14, 18, 20, 21, 38). The M protein thus is initially a soluble cytoplasmic protein and then subsequently associates with cellular membranes. The specific sorting of M protein would therefore be mediated by very different cellular mechanisms than that of

1. Abbreviation used in this paper: RER, rough endoplasmic reticulum.

the integral membrane glycoproteins studied to date. We have therefore undertaken to examine the distribution of M protein in VSV-infected MDCK cells while controlling the intracellular distribution of the G protein.

Materials and Methods

Cell and Virus Growth

MDCK cells were cultured in DME (Gibco Laboratories, Grand Island, NY) containing 10% FBS (Gibco Laboratories). Before an experiment, the cells were plated at a density of $3 \times 10^5/\text{cm}^2$ on 35-mm dishes (Falcon Becton Dickinson, Oxnard, CA) either with or without No. 1.5 cover glass and cultured for 5 d to allow them to establish polarity.

Polarized MDCK cells were infected with VSV at a multiplicity of 50 plaque-forming units (titered on *Vero* cells) per cell in 0.1 ml of medium containing 50 μg per milliliter DEAE dextran (500,000 D, Pharmacia Fine Chemicals, Piscataway, NJ). After a 30-min adsorption period at 32°C, the inoculum was removed and the cells were fed with fresh growth medium and incubated at 40°C for the times indicated in the figure legends. In temperature shift experiments, cycloheximide (20 $\mu\text{g}/\text{ml}$) was added to the infected cells just before the temperature shift to prevent further protein synthesis. Similar results were obtained if the cycloheximide was omitted.

Radiolabeling and Immunoprecipitation of VSV Proteins

Confluent, polarized monolayers of MDCK cells were prepared and infected as described above except that no cover glass was used. After the adsorption period, the cells were fed with 1 ml of minimal essential medium without methionine (Flow Laboratories, McLean, VA) containing 1 μl of DME medium (Gibco Laboratories), 20 mM Hepes, pH 7.2, 5% dialyzed FBS (HyClone Laboratories, Logan, UT), and 50 μCi [^{35}S]methionine (ICN Laboratories, Plainview, NY). After incubation for the times indicated in the text, the cells were lysed in 1 ml PBS (150 mM NaCl, 10 mM sodium phosphate, and 10 mM potassium phosphate pH 7.5) with 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 0.5% sodium deoxycholate (Sigma Chemical Co.), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co.). The nuclei were removed by centrifugation for 1 min in a microfuge (Eppendorf, model No. 5414). The supernatant was adjusted to 0.3% SDS, 12 $\mu\text{g}/\text{ml}$ affinity purified rabbit anti-VSV G protein (2), 12 $\mu\text{g}/\text{ml}$ of monoclonal anti-VSV M protein (see below), 0.1 mM PMSF, and 0.5% sodium azide. The antibodies were allowed to bind to their antigens for 16 h at 0°C. Nonspecific aggregates were removed by centrifugation at 19,000 rpm for 20 min in a Sorvall SS-34 rotor and 20 μg of affinity purified rabbit anti-mouse IgG was added to the supernatants. After incubation for an additional 60 min at 0°C, 50 μl immunoprecipitin (Bethesda Research Labs, Bethesda, MD) was added. After a 30-min incubation at 0°C, the immunoprecipitin was collected by centrifugation for 1 min at 13,000 g in a microfuge. The immunoprecipitates were washed and analyzed by PAGE followed by radioautography as previously described (49). Labeling of the G and M proteins was determined by densitometric scanning of the radioautograms.

Sucrose Gradient Analysis of Membrane-bound M Protein

Cells were infected and labeled as described above. 60 min after infection, the monolayer was rinsed two times with 1 ml of homogenization buffer (10% sucrose, 1 mM EDTA, pH 7.4, 0.2 mM PMSF). The cells were harvested with a rubber policeman in 2 ml of homogenization medium and subjected to 10 strokes with a tight-fitting dounce homogenizer (Wheaton Instruments, Melville, NJ). The nuclei were removed by centrifugation at 1,000 rpm for 4 min. The supernatant was adjusted to 80% sucrose (wt/vol) and placed in the bottom of an SW41 centrifuge tube (Spinco, Palo Alto, CA). The homogenate was overlaid with 6 ml 65% sucrose (wt/vol) in water followed by 3 ml 10% sucrose in water. The gradient was spun at 5°C for 16 h at 35,000 rpm. An Isco gradient fractionator was used to collect 1.5 ml fractions. Before immunoprecipitation, each fraction was diluted with 6 ml of RIPA (1% Triton X-100, 0.4% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.8). Quantification of M protein was carried out as described above.

Preparation and Characterization of Monoclonal Antibodies

Balb/c mice were immunized with 100 μg ultraviolet killed ts045 per injection as follows: the initial immunization was subcutaneous and intramuscular in complete Freund's adjuvant. At 4-wk intervals additional intravenous injections were administered via the tail vane. This procedure was continued until the titer of antibody to VSV in the serum was sufficient to detect a positive reading in an ELISA assay at a serum dilution of 10^{-5} (see below). After a period of at least 4 wk after the previous injection, a final intravenous injection was performed. 4 d after the final injection, the mouse was sacrificed, the spleen was removed and the resident lymphocytes were forced through a fine stainless steel mesh. The dissociated cells were fused with mouse myeloma cell line SP2/0 using 0.5 ml 50% polyethylene glycol 4,000 (Merck & Co., Inc. Rahway, NJ) in DME for 90 s, the fusion medium was diluted over a 10-min period with 10 ml DME, and the cells were further diluted into HAT selective medium (containing 15% FBS, 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) to a concentration of $2 \times 10^6/\text{ml}$. The fused cells were mixed with 2 vol of HAT medium containing 10^6 normal spleen cells per ml (as a feeder layer), and 0.15 ml of this final mixture was plated per well in 96-well microtiter dishes. As colonies became visible, samples of the culture supernatants were screened by ELISA and positive cultures were expanded into individual wells of 24-well plates. The specificity of the antibodies present in the supernatants of these cultures was analyzed as in Fig. 1 using SDS-polyacrylamide gel purified VSV proteins that had been transferred to nitrocellulose paper (2). Only 50% of the ELISA positive supernatants reacted with any of the viral proteins on nitrocellulose. The cells secreting antibodies that could be easily characterized in this manner were recloned, grown up in mass culture, and injected into the peritoneum of pristane-primed Balb/c mice. The resulting ascites culture was harvested and the antibodies were purified by ammonium sulfate fractionation and DEAE cellulose chromatography. Antibodies were stored at -20°C in PBS containing 50% glycerol.

ELISA Screening of Hybridomas

VSV was harvested from culture medium by centrifugation for 30 min at 30,000 rpm in a Beckman 60Ti rotor. The virus pellet was resuspended in PBS, inactivated by exposure to UV radiation, added to 96-well microtiter plates (0.1 μg of virus per well), and incubated at room temperature for 2 h. Unbound virus was washed out in PBS and the bound virus was rendered permeable with 1% Triton X-100 in PBS. Nonspecific binding sites on the plates were blocked with 3% BSA in PBS for an additional 2 h. The hybridoma supernatants were then added to the pretreated wells, incubated for 2 h at room temperature, and washed out with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). Goat anti-mouse IgG coupled to alkaline phosphatase (Miles-Yeda) was diluted 1:1,000 in 3% BSA in PBS was added to the wells, incubated for 2 h at room temperature, and washed out with Tris-buffered saline. The plates were developed with 0.2 mg/ml 4-methylumbelliferyl phosphate (Sigma Chemical Co.) in 1 M Tris-HCl, pH 8.0. The plates were examined under ultraviolet light and photographed with a polaroid copy camera.

Indirect Immunofluorescence Microscopy

Fixation, extraction, and monoclonal antibody staining of cells was carried out as previously described (4) except that the secondary antibody used was biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA). The secondary antibody was visualized using a third staining with a 1:200 dilution of Texas red-conjugated streptavidin (Bethesda Research Laboratories). The stained cells were examined with a Leitz Dialux microscope equipped with L₃ and N fluorescence cubes. Photomicrographs were taken with a Leitz Orthomat 35-mm camera.

Horseshoe Peroxidase Immunocytochemistry

Cells were grown directly in the culture dishes (no cover glass was used). The fixation and staining were carried out as described above except that an avidin/biotin-horseshoe peroxidase reagent mix (Vector Laboratories) was substituted for the streptavidin/Texas red. The samples were washed for 20 min with PBS, and incubated for 30 min in a solution containing 1 mg/ml diaminobenzidine, 100 mM sodium phosphate, pH 7.4, and 0.01% H₂O₂. The cells were then dehydrated with ethanol and embedded in Epon 812. Sections (0.3–0.5 μm) were cut perpendicular to the cell monolayer and

mounted on glass slides with PermMount (Fisher Scientific, Pittsburgh, PA) under No. 1.5 glass cover glass.

Results

Characterization of Monoclonal Antibodies

Monoclonal antibodies were raised and purified as indicated in the Materials and Methods section. The specificity of these antibodies is demonstrated in Fig. 1. Lysates of 3×10^5 ts045 or mock-infected MDCK cells were subjected to SDS-PAGE and the proteins were then electrophoretically transferred to nitrocellulose paper. The paper was probed with antibodies to the M protein (lanes 1 and 2) or to the G protein (lanes 3 and 4). The antibodies reacted only with proteins of the appropriate molecular weight in infected cells (lanes 1 and 3) and did not react with any antigen from uninfected cells (lanes 2 and 4, respectively).

Newly Synthesized M Protein Reaches the Plasma Membrane Independently from the G Protein

In preliminary experiments, the distribution of the M and G proteins was examined in MDCK cells infected with wild-type VSV. As expected, the G protein was seen in the Golgi apparatus and on the plasma membrane. The M protein was also seen on the plasma membrane, but not associated with the Golgi apparatus (not shown). However, under these conditions, it was difficult to distinguish staining of the nuclear envelope or any discrete elements of the RER. Thus, although the G protein was presumably associated with the RER, we could not determine whether the cytosolic distribution of the M protein was due to M associated with the RER or to M protein free in the cytosol.

To overcome these difficulties and to "uncouple" the pathways of M and G protein biogenesis, we made use of ts045. At the nonpermissive temperature, 40°C, the G protein of this temperature-sensitive mutant is synthesized normally, but is unable to leave the endoplasmic reticulum (2, 4, 25, 27). Under these conditions, labeling of G protein in the nuclear envelope is quite evident and any association of the M and G proteins in the RER could be resolved. In addition, after shifting infected cells to the permissive temperature (32°C), the G protein is able to move normally to the cell

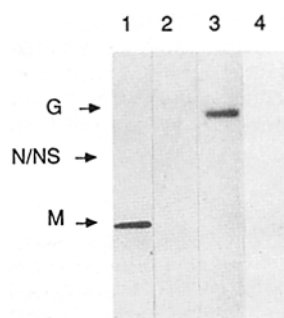


Figure 1. Characterization of monoclonal antibodies to the VSV M and G proteins. VSV proteins were separated on SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes as previously described (2). Lanes 1 and 3 contain extracts of ts045 infected MDCK cells. Lanes 2 and 4 contain extracts of mock-infected MDCK cells. Lanes 1 and 2 were probed with monoclonal anti-M protein. Lanes 3 and

4 were probed with monoclonal anti-G protein. The mobility of the G, N/NS, and M proteins were determined from samples of purified VSV run in parallel on the same gels.

surface. At both temperatures, the M protein functions normally. Thus, the intracellular distribution of the M protein can be examined while manipulating the location of the G protein.

We began by comparing the distributions of the M and G proteins in ts045-infected MDCK cells that had been incubated at the nonpermissive temperature. Cultures were fixed and subsequently stained with either monoclonal antibody to the G or M proteins (Fig. 2, A and B). As expected, the G protein was restricted to the RER including the nuclear envelope (*small arrowheads* in Fig. 2 A). In marked contrast, the M protein was found either free in the cytoplasm or associated with the plasma membrane (*arrow* in Fig. 2 B). In contrast to the labeling of the G protein, there was no apparent labeling of the nuclear envelope. No labeling of any other intracellular membrane structure could be detected. The M protein is therefore able to associate with the plasmalemma independently from the G protein. In addition, the M protein does not associate efficiently with the G protein held in the RER at the nonpermissive temperature.

To insure that the M protein localized in Fig. 2 was not derived from the initial virus inoculum, we adsorbed ts045 onto MDCK cells as in Fig. 2. After the adsorption period, the cells were incubated either in growth medium (Fig. 3 A) or in growth medium containing 20 μ g/ml cycloheximide to prevent the de novo synthesis of viral proteins (Fig. 3 B). After incubation for 1 h at 40°C, the cells were fixed and stained as in Fig. 2 B. M protein was only detected in cells that had been allowed to synthesize viral proteins. We conclude that newly synthesized M protein is able to associate specifically with the plasma membrane of MDCK cells and that this association does not require the presence of G protein in that membrane.

The M Protein Fails to Bind to the G Protein on the RER or the Golgi Apparatus

We next wished to examine whether at the permissive temperature the M protein could associate with the G protein on internal membranes. Accordingly, cells infected as in Fig. 2, A and B, were shifted to 32°C for 10 min. As we had previously shown, this time period is sufficient for the G protein to reach the Golgi apparatus, but not sufficient for it to reach the cell surface (2, 3, 4) (Fig. 2 C). In addition, some G protein still remains in the RER at this time (Fig. 2 C). Again, the M protein is restricted to the cytosol and plasma membrane of these cells. No labeling of either the RER or Golgi apparatus containing G protein was seen (Fig. 2 D). 50 min after the temperature shift, G protein was seen on the basolateral plasma membrane, but was no longer detected on the nuclear envelope (Fig. 2 E). The distribution of the M protein remained unchanged (Fig. 2 F). Apparently, the presence of G protein on internal membranes is not sufficient to induce the association of M protein with those membranes even at the permissive temperature. Conversely, the arrival of G protein at the plasma membrane does not apparently alter the subcellular distribution of the M protein. Thus, the M protein associates with the plasma membrane both in the presence and absence of G protein.

Quantification of VSV Membrane Proteins in Infected MDCK Cells

Metsikko and Simons have shown that the spikeless VSV

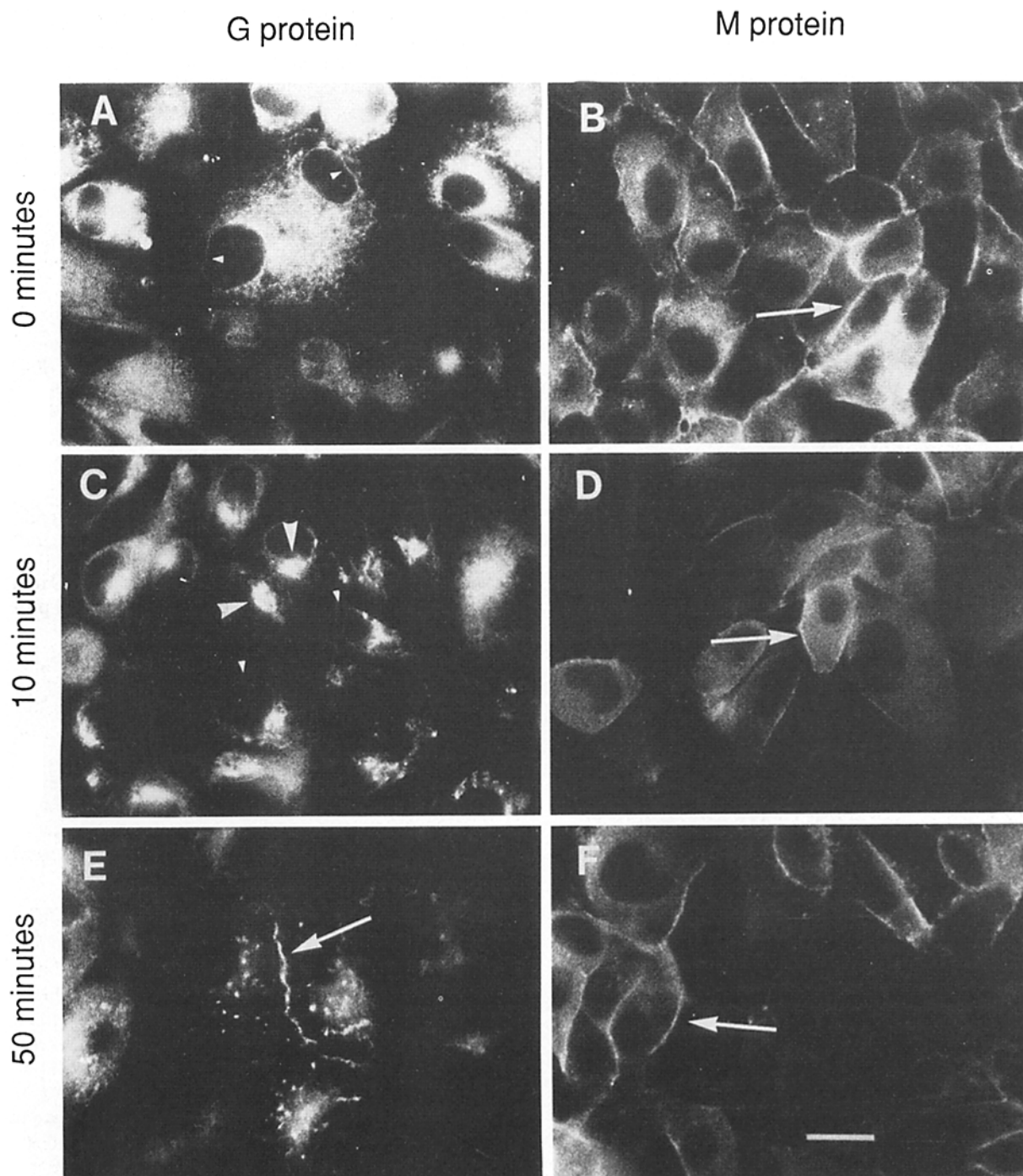


Figure 2. Distribution of the VSV G and M proteins in ts045-infected MDCK cells. MDCK cells were infected with ts045 and incubated at 39.9°C for 1 h. Cells in *A* and *B* were immediately fixed. Cycloheximide was added (20 µg/ml) and the cells were shifted to 32°C for 10 min (*C* and *D*) or 50 min (*E* and *F*). (*A*, *C*, and *E*) Distribution of the VSV G protein. (*B*, *D*, and *F*) Distribution of the VSV M protein. In *A* and *C* small arrowheads indicate staining of the nuclear envelope. In *C* large arrowheads indicate labeling of the presumptive Golgi apparatus. Arrows indicate examples of lateral plasma membrane labeling in *B*, *D*, *E*, and *F*. Bar, 20 µm.

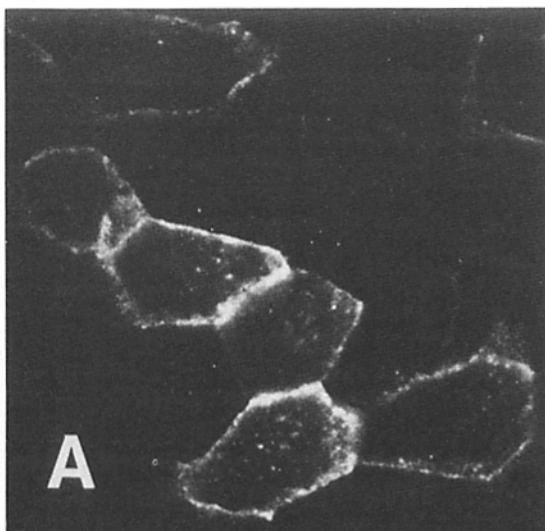
particles that bud from infected *Vero* cells late in infection incorporate "G tails" (36). These tail fragments are derived from the carboxy terminus of G and contain the transmembrane and cytoplasmic domains of the G protein (23, 36). They arise when an endogenous protease clips the G protein and releases its ectodomain as a soluble fragment G_s (8, 23,

30, 36). It is therefore possible that the M protein is directed to its target membrane by these tail fragments.

We therefore quantified the number of such fragments that might potentially bind M protein to the plasma membrane. Cells were infected with ts-045 and labeled continuously at 40°C with [³⁵S]methionine. At 30, 60, and 90 min post-

M Protein

- Cycloheximide



+ Cycloheximide

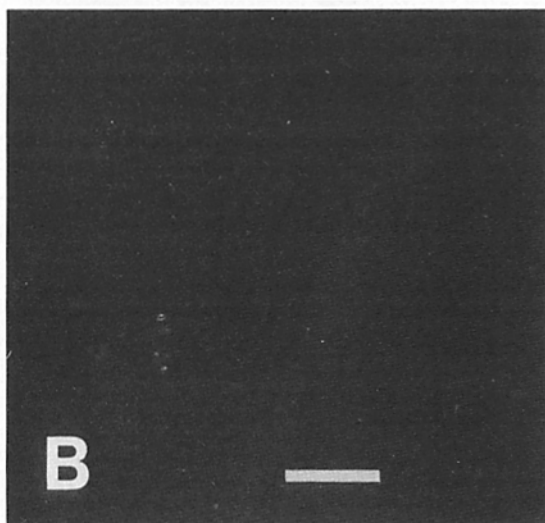


Figure 3. Role of de novo protein synthesis in the labeling of M protein. MDCK cells were infected and labeled as in Fig. 2 *B* except that after adsorption of virus, the cells were incubated either in growth medium (*A*) or in growth medium + 20 μ g/ml cycloheximide (*B*). The time of photographic exposure and the conditions of printing the negatives were identical for *A* and *B*. Bar, 20 μ m.

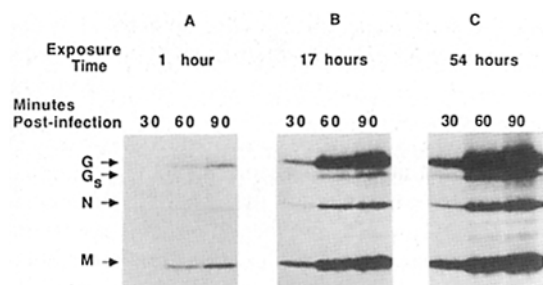


Figure 4. Quantification of M and G_s in ts045-infected MDCK cells. After a 30-min adsorption period at 30°C, MDCK cells were labeled continuously with [35 S]methionine for 30, 60, or 90 min. After the appropriate incubation time, the cells were solubilized and the M and G proteins were immunoprecipitated and separated by SDS-PAGE. The gels were dried and then exposed to X-ray film for 1 h (*A*), 17 h (*B*), or 57 h (*C*).

fection, the labeling medium was harvested and cell extracts were prepared. The G and M proteins were immunoprecipitated from the cells and from the labeling medium. We analyzed the immunoprecipitates by SDS-PAGE. Although the G, G_s , and M proteins were readily evident in precipitates of the cell extracts, they were not seen in the precipitates from the labeling medium. Fig. 4 shows radioautograms resulting from three exposures of the cell extract immunoprecipitates. Although the tail fragment is not directly displayed on these gels, G_s is clearly resolved. We therefore used G_s to indirectly quantitate the "tail" fragment. It is readily evident that both 30 and 60 min after infection, there is far less G_s than M protein. Densitometric analysis of these radioautograms reveals that at 30 and 60 min after infection, there is, respectively, only 0.13 and 0.12 times as much G_s as M protein in the cell (Table I). In addition we have observed that G_s is largely sensitive to endoglycosidase H (not shown). Thus, the cleavage of G is occurring primarily in the endoplasmic reticulum and the tail fragments would also be originating in the endoplasmic reticulum. Assuming that the tail fragments are able to leave the RER and require only 30 min to reach the cell surface, the number of fragments at the cell surface 60 min after infection would be the same as the number in the endoplasmic reticulum 30 min after infection. This represents 2% of the number of M proteins in the cell 60 min after infection.

To determine the fraction of M protein associated with the plasma membrane, cells were labeled as in the experiment shown in Fig. 4. 60 min after infection, the cells were harvested, homogenized, and applied to the bottom of a sucrose step gradient as described in the Materials and Methods section. The cellular membranes would be expected to float and to accumulate at the interface between the 65 and 10% sucrose steps. Indeed an opalescent band was seen at the position of this interface. Equal 1.5-ml fractions were collected and the M protein was immunoprecipitated and analyzed by SDS-PAGE. Fig. 5 shows a radioautogram of the dried gel.

Table I. Quantification of M and G_s in MDCK Cells 30 and 60 Min Postinfection

Time postinfection	G_s	M	G_s/M
30 min	14/1.6	132 (12)	0.11 (0.13)
60 min	79 (8.8)	800 (73)	0.10 (0.12)

The densitometric data for G_s at 30 and 60 min postinfection came from Fig. 4, *C* and *B*, respectively. The densitometric data for M protein came from Fig. 4 *A*. The data, in arbitrary units, were corrected for exposure time. In addition, we wished to correct for the relative number of methionine residues in the G_s and M proteins. The M and G genes code for 11 methionines each (50). The initiator methionine of the G protein is removed co-translationally along with its signal sequence (22, 29). In addition, we used the following considerations to estimate the number of methionine residues lost by G_s on its cleavage from the G protein. The migration difference between G and G_s would normally indicate a shift in molecular weight of 6,000 D (\sim 54 amino acids) (10, 17, 23). However, we have shown that the transmembrane domain of the G protein does not contribute to the apparent molecular weight on SDS-polyacrylamide gels (49). We therefore estimate that G_s is actually \sim 74 amino acids shorter than G. The 104 carboxyterminal amino acids contain only 1 methionine. We have therefore used an estimate of nine methionine residues in G_s to calculate the molar ratios of M and G_s in Table I. The first number in each column represents the area under the peaks of the densitometric scans of G_s and M. The second number (in parentheses) represents the calculated mole fraction based on division of the area by the assumed number of methionine residues in each protein.

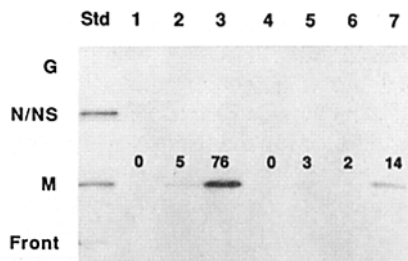


Figure 5. Sucrose gradient analysis of M protein membrane association. Polarized monolayers of MDCK cells were infected with ts045 and incubated in labeling medium for 1 h. Homogenates were prepared and spun at 35,000 rpm on a sucrose step gradient as described in the Materials and Methods section. The M protein was immunoprecipitated from each of the seven fractions, and subjected to PAGE. (Fraction 1) Top of the gradient; (fraction 3) the interface between the 10 and 65% sucrose layers; (fraction 7) the bottom of the gradient where the lysate was loaded. The numbers above each band refer to the percent of the total labeled M protein found in that band.

M protein is seen primarily in the bottom fraction (fraction 7) and at the interface between the 10 and 65% sucrose steps (fraction 3). The results of densitometric analysis are summarized in the numbers above each band. Fractions 2 and 3 contained 81% of the M protein. Thus, if the G tail were responsible for bringing the M protein to the membrane, each molecule of G tail would have to organize at least 40 molecules of M protein.

Polarity of M Protein on the Plasma Membrane

Having established that the M protein is segregated onto the plasma membrane of polarized MDCK cells, we wished to determine whether it associates specifically with either the basolateral or apical plasma membrane domains of this epithelium. We again infected the MDCK cells with ts045 and incubated them at 40°C. 90 min after infection, we fixed the cells and immunostained for M protein using indirect horseradish peroxidase immunocytochemistry (see Materials and Methods). The stained cells were dehydrated, embedded in epon 812, and sectioned in a plane perpendicular to the monolayer. Fig. 6 shows the distribution of M protein within the MDCK cells. Again, of all the cellular membrane systems, only the plasma membrane exhibits any affinity for the M protein. In general, the M protein was restricted to the basolateral plasmalemma with the strongest labeling along the lateral membranes (*arrowheads*). However, there were always a number of cells in which the M protein was uniformly distributed over the entire plasmalemma (*solid circles*). These cells had apparently lost their polarity. Such cells serve as an internal control that the immunocytochemical procedures we employed can demonstrate apical as well as basolateral staining of M protein. We conclude that even when the G protein is restricted to the RER, M protein is able to associate specifically with the basolateral plasma membranes of polarized MDCK cells.

Discussion

Integral membrane proteins destined for transport to the

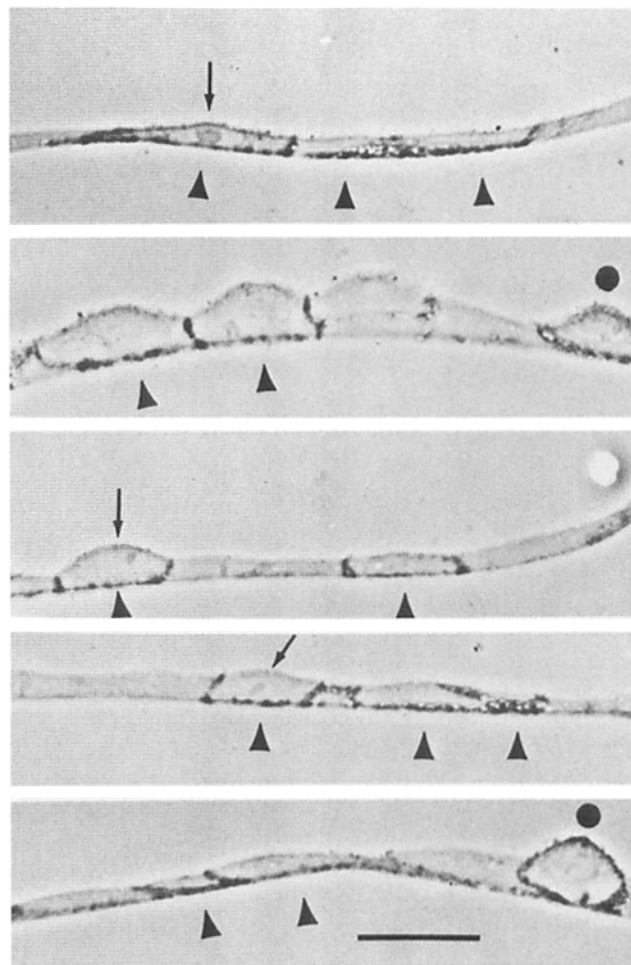


Figure 6. Distribution of the VSV M protein in ts045-infected MDCK cells. MDCK cells were infected with ts045 and incubated at 39.9°C for 1 h and fixed in 3% paraformaldehyde in PBS. M protein was labeled using indirect horseradish peroxidase immunocytochemistry (see Materials and Methods), dehydrated and embedded in Epon 812. Semithin sections were cut perpendicular to the cell monolayer. Basolateral staining of infected cells is indicated with arrowheads. Solid circles mark nonpolarized cells. Bar, 20 μ m.

plasmalemma are known to contain signals which direct them to the RER (28, 29). These signals are generally removed co-translationally and other signals must therefore be responsible for subsequent intracellular sorting. Here we have shown that the M protein which is made on free polyosomes (14, 18, 20, 21, 38) shares with integral membrane proteins the ability to be sorted with a high degree of specificity. Previous studies have demonstrated M protein in the cytoplasm and at the plasma membrane of VSV-infected cells (42, 43). Our work extends those studies by demonstrating that the M protein associates with the basolateral plasmalemma of polarized MDCK cells and that this association is independent of the intracellular distribution of the G protein. Biochemical investigations have shown that the M protein associates with its target membranes within 2–3 min after synthesis in the cytosol (1, 13, 26). It is therefore evident that the targeting of the M protein must be very different from the deliberate transport and sorting of integral proteins made on the RER.

The mechanism of this targeting remains unknown. Perhaps the most appealing hypothesis had been that the M protein does not interact directly with membranes, but simply binds to the cytoplasmic domain of the G protein at the target membrane (42) or that it binds indirectly to the G protein via nucleocapsids (41). Support for these models came from cross-linking studies in which the M protein could be specifically cross-linked to the G protein in virions, thus demonstrating a specific interaction between M and G (15, 40). Further, although M protein can be cross-linked to virion lipids and to lipophilic reporter molecules (33, 44, 58), reagents such as 16-azido-³H-palmitic acid and 4,4'-dithiobisphenylazide which react with proteins only in the hydrophobic core of the lipid bilayer fail to react with the M protein (55, 58). Thus, in virions, the M protein appears to be bound to the G protein and to occupy a close yet peripheral position vis-a-vis the lipid bilayer. The proposed interaction of M protein with membranes solely via the G protein provides a rationale as to how these two membrane proteins might coordinately reach a common destination. Support for such a model was weakened by *in vitro* reconstitution experiments in which the M protein was reported to bind to isolated membranes of uninfected cells (11, 12, 39). However, such association of M protein with host membranes was not seen in all systems and its physiological significance was questioned (26). The experiments described here provide a direct test of this model. Under conditions in which the G protein is restricted to the RER of polarized MDCK cells, the highest concentration of M protein is found on the basolateral plasmalemma not the RER. It is thus evident that the M protein becomes associated with basolateral plasmalemma independently of the G protein.

Although we could not detect G protein on the cell surface in these experiments, it was possible that a fragment of the G protein that includes the transmembrane and cytoplasmic tail of the G protein might have been responsible for the observed localization of M protein (36). To minimize this possibility, we localized the M protein in cells infected for only 60 min. As the G protein requires ~30 min to move from the RER to the plasma membrane, we estimated that only those G tails that leaked out of the RER 30 min after infection would have had time to reach the plasma membrane by 60 min postinfection. Quantitation revealed that there could have been no more than 2% as much G tail on the plasma membrane as M protein in the cell 60 min postinfection (Fig. 4 and Table I). At this time 81% of the M protein floats to the interface between 10 and 65% sucrose and is presumably membrane bound. Thus, each G tail would have to direct at least 40 molecules of M protein to the basolateral plasma membrane.

Odenwald et al. reported that during wild-type virus infection, the M protein was seen on the plasma membrane only in association with nucleocapsids. However, three observations indicate that viral nucleocapsids are not responsible for the M protein localization reported on here. First, during infection with most M protein mutants, only G and M containing particles devoid of nucleocapsids are produced. Second, Ono et al. reported that the mutant M protein of tsG33 aggregates intracellularly at the nonpermissive temperature and induces intracellular budding (43). Finally, using a late SV-40 promoter, we have expressed the M protein in nonpolarized COS-1 cells (Florkowitz, R., J. E. Bergmann, and

J. K. Rose unpublished observations). Under these conditions, the M protein also becomes associated with the plasmalemma despite a total lack of G protein in the cells. It is therefore highly unlikely that the observed localization of M protein is due to any other VSV protein including G protein or its membrane anchor.

Three formal mechanisms can be proposed for the observed specificity of M protein localization. First, the M protein may associate specifically with lipid components found in the basolateral plasmalemma. Second, the M protein may associate stably with the cytoplasmic domains of the host cell's basolateral plasma membrane proteins. Third, the M protein may become membrane associated due to some as yet unidentified posttranslational modification, if the relevant enzyme(s) were restricted to the basolateral plasmalemma, the M protein would likewise become restricted to that membrane.

Possible Association of M Protein with Lipids

Several investigators have examined the interaction of the VSV M protein with lipid. In addition to the cross-linking studies described above, Zakowski and co-workers (59) have examined the binding of M protein to reconstituted phospholipid vesicles. They were able to show that the M protein could be reconstituted into negatively charged, but not neutral vesicles. High salt prevented this reconstitution, but addition of salt to the vesicles after reconstitution did not elute M protein from the vesicles. This data supports the hypothesis that the M protein may associate posttranslationally with the lipid constituents of membranes. However, although the overall lipid composition of the apical and basolateral plasma membranes differs (7, 24, 57), the cytoplasmic leaflet of these domains is probably the same (56). It is therefore unlikely that the M protein associates specifically with the basolateral plasma membrane due to its lipid composition.

Possible Association of M Protein with the Cytoplasmic Domain of Basolateral Plasma Membrane Proteins

Evidence from several laboratories indicates that the cytoplasmic domain of the VSV G protein may be necessary (19, 46) if not sufficient (35, 52) for the polarized delivery of G protein to the basolateral plasma membrane of MDCK cells. If components of the cellular sorting machinery recognize features of the cytoplasmic domain of the G protein, it is likely that other basolateral membrane proteins of MDCK cells share these features. As the physiological role of M protein in virion assembly requires that it bind to the cytoplasmic domain of the G protein, it is reasonable that it might similarly bind to the corresponding domains of a broad spectrum of proteins found in the basolateral plasma membrane. Indeed, there are reports that the VSV M protein and nucleocapsids can form virus like particles at least partially coated by cellular membrane proteins (9, 32, 36, 53, 60). Although attractive, there is as yet no direct evidence supporting such a mechanism of M protein targeting.

Possible Role of Posttranslational Modification in the Targeting of M Protein

The above proposal that M protein associates with the basolateral plasmalemma via host proteins presents one con-

ceptual problem. How does the M protein preferentially switch to plasmalemmal G protein during virus budding yet not bind to the G protein that resides in the RER membranes? If the M protein were posttranslationally modified at its target membrane in such a way that it became strongly associated with that membrane, the specificity of the targeting could be explained by the location of the modifying enzyme. After modification, M would remain associated with the membrane, but not with the enzyme. M protein would therefore be free to bind to the G protein when G reached the same membrane. Again, there is no evidence supporting this model.

In summary, data have been presented which demonstrate a novel type of intracellular targeting of the VSV matrix protein. After synthesis on free ribosomes, the M protein binds directly to the basolateral membrane of MDCK cells. This specific membrane association is not mediated by the VSV G protein.

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